ADENOSINETRIPHOSPHATASE ACTIVITY OF SQUID MANTLE MUSCLE (LOLIGO PEALII) [1, 2]

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Studies on the biochemistry of vertebrate striated muscle clearly indicate the presence of two distinct enzymes which catalyze the hydrolysis of the terminal phosphate from adenosinetriphosphate (ATP). One adenosinetriphosphatase (ATPase) is calcium-activated and appears to be identical with myosin (Engelhardt, 1946; Szent-Gyorgyi, 1947). The second ATPase is magnesium-activated and is associated with the particulate matter of the cell (Kielley and Meyerhof, 1948; Perry, 1952; Kitiyakara and Harman, 1953; de Villafranca, 1954). Although it has not been clearly established, most workers assume that myosin-ATPase and the contraction phenomenon must be closely associated. A contribution to the understanding of the enzymes' role, as well as to the general understanding of muscular contraction, could be made by a careful examination of their distribution in muscle tissue from various members of the animal kingdom.

Very few data on muscle ATPase from invertebrates have been published. Humphrey (1949) concluded that there is both a myosin-ATPase and a water-soluble-ATPase in the adductor muscle of the oyster, *Saxostrea commercialis*. More recently Gilmour and Calaby (1952) report two ATPases present in locust muscle (*Locusta migratoria* and *Gastrimargus musicus*); one a calcium-activated, myosin-ATPase and the second, much more concentrated, a magnesium-activated, water-soluble-apyrase. The latter differs from that found in mammalian muscle in not being associated with the particulate matter of the cell.

The large quantity of easily available muscle in the mantle of the squid makes it a highly desirable organism for a comparative study of the biochemistry of muscle. The present paper deals with an attempt to locate and isolate the ATPase activity in the mantle muscle of the squid, *Loligo pealii*. A preliminary account of this work has been published (de Villafranca, 1953).

Materials and Methods

Fresh squid were decapitated and the mantle placed on ice. After removing the fins and pen, the epidermis was stripped from the remainder of the mantle. The mantle was then washed with distilled water and strips, about 5 mm. wide, were cut into 10 volumes of ice cold distilled water and homogenized for one minute in a Waring Blendor run at top speed. The homogenate was centrifuged twice, first at 2300 rpm for 5 minutes and then the supernatant at 4600 rpm (up and

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down) thus separating the homogenate into a water-soluble extract (S_1) and into a residue of debris and water-insoluble material. The combined residues of the two centrifugations (2300 rpm and 4600 rpm) were added to 10 volumes of cold 0.5 *M* KCl to extract the myosin and the KCl homogenate was centrifuged at 2300 rpm for 5 minutes. Precipitation of the myosin by diluting the KCl extract with 6 volumes of cold distilled water was performed twice.

Throughout the extraction the material was kept as cold as possible in ice water baths. Centrifugations were carried out at room temperature. The pH was maintained above neutrality, approximately pH 7.4, by the addition of solid sodium bicarbonate when necessary.

ATPase activity of the various fractions was determined by measuring the increase in inorganic phosphorus after ten minutes incubation at 25° C. of duplicate samples each containing, in addition to the tissue, approximately 0.002 *M* ATP, 0.05 *M* histidine buffer at pH 5.9, 0.05 *M* KCl, and 0.01 *M* $CaCl_2$ or some other ion as specified. The total volume of the reaction mixture was one ml. The reaction was started by the addition of substrate and stopped by trichloroacetic acid (TCA) to a final concentration of 5%. The inorganic phosphorus was determined on the TCA supernate in a Bausch and Lomb colorimeter at 660 mμ according to the method of Fiske and Subbarow (1925), and the enzyme activity expressed as Q_P (Bailey, 1942); *e.g.*, microliter equivalents of phosphorus hydrolyzed in an hour by one mg. of tissue protein.

Protein was estimated by multiplying nitrogen values, obtained by semi-micro Kjeldahl distillation and titration, by the factor 6.25. The tissue proteins were first precipitated with TCA and then digested with a selenium, copper, sulfuric acid mixture before the distillation.

ATP was purchased as the sodium salt (chromatographically pure) from the Schwarz Laboratories, New York City. Inosine-triphosphate (ITP) was a gift from Dr. H. M. Kalckar.

Results

The data from a typical experiment, summarized in Table I, indicate the relative lack of water-soluble ATPase in squid mantle muscle. In this particular experiment the water-soluble fraction (S_1), although containing 18.5% of the mantle protein nitrogen, contains only 0.9% of its ATPase activity. Microscopic examination of this fraction reveals a homogeneous suspension of particles which are, presumably, the mitochondria. Numerous attempts were made to obtain a second ATPase (in addition to a myosin-ATPase) by extracting mantle with molar sucrose, or 0.5 *M* KCl, and following this extraction with differential centrifugation at 20,000 g and 100,000 g to spin down the mitochondria and microsomes, but the results were essentially negative (de Villafranca, 1953). Mitochondrial preparations obtained from sucrose extracts did have somewhat higher proportions of ATPase activity, but these preparations exhibited the same ion activation and pH optimum as the myosin-ATPase. Furthermore, the bulk of the activity could be removed from this type of preparation by washing with 0.5 *M* KCl. The ATPase activity of the soluble fraction (including the particulate matter) was thus considered to be due to myosin-ATPase contamination and not due to an inherent particulate ATPase.

In contrast to the S_1 fraction, the 0.5 *M* KCl extract (MS) of the water-insolu-

TABLE I

The ATPase activity of fractions isolated from squid mantle muscle

Fraction	Q_P	Percentage activity	Percentage nitrogen
Whole homogenate	1140	100.0	100.0
Water-sol. ext. (S_1)	54	0.9	18.5
Residue after KCl ext.	1335	7.6	6.5
KCl extract (MS)	1558	92.3	68.1

ble material contains most of the mantle ATPase activity (Table I). It was from the MS fractions that myosin was precipitated by dilution with 6 volumes of distilled water.

In Figure 1 may be seen the effect of magnesium, manganese, or calcium ions on the ATPase activity of twice-precipitated myosin. Only calcium activates the enzyme to any extent, activating optimally at a concentration of 0.01 *M* $CaCl_2$ both at pH 5.9, as is illustrated, or at pH 8.93. Manganese activates slightly while magnesium is without effect. ATPase activity in the presence of 0.02 *M* $CaCl_2$ was found to be optimal at 0.05 *M* KCl but other concentrations of KCl, up to 0.1 *M*, were only slightly less effective.

The curve illustrating the relationship between myosin-ATPase activity and pH is shown in Figure 2. A definite optimum exists in the region of pH 5.9 with

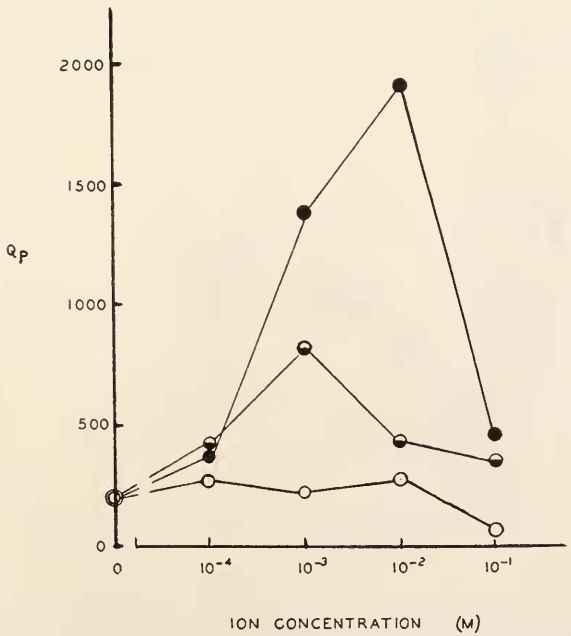

FIGURE 1. The effect of different concentrations of calcium, magnesium, or manganese on the ATPase activity of twice-precipitated squid myosin. Activity was measured in 0.05 *M* histidine buffer (pH 5.9), 0.05 *M* KCl, 0.002 *M* ATP, and the ions as indicated: $CaCl_2$, ●; $MnCl_2$, ◒; and $MgCl_2$, ○.

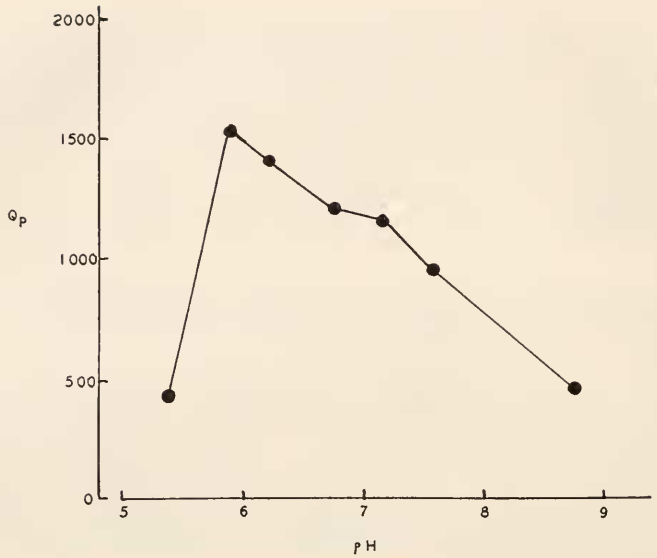

FIGURE 2. ATPase activity of twice-precipitated myosin as a function of pH. Activity measured in 0.05 *M* histidine, 0.02 *M* $CaCl_2$, 0.05 *M* KCl, and 0.002 *M* ATP.

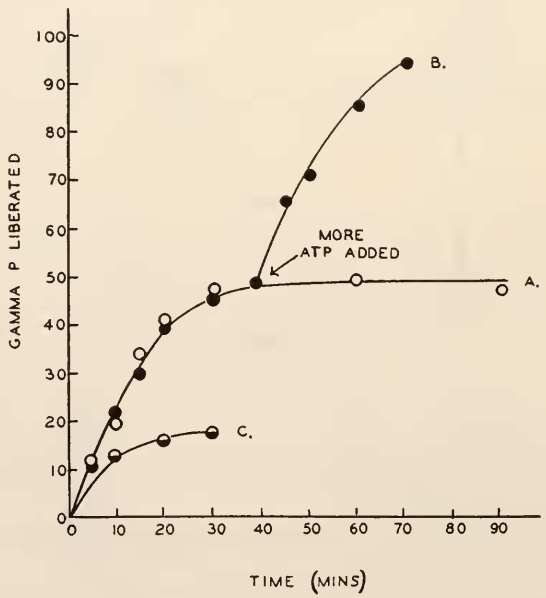

FIGURE 3. Rate of hydrolysis of ATP and ITP by twice-precipitated myosin. In experiment A, 124 gamma of "7 min." P were present; experiment B, 147 gamma and an additional 147 gamma of "7 min." P, aliquot of ATP was added as indicated. Experiment C had ITP, 123 gamma of "7 min." P, as substrate. Activity in all the experiments was measured in 0.05 *M* histidine buffer (pH 5.9), 0.01 *M* $CaCl_2$, and 0.05 *M* KCl.

much greater activity than at pH 8.8 or higher. The most alkaline pH at which the activity was measured was pH 9.4. In this case the activity was proportionally lower than that shown at pH 8.8 (Fig. 2).

That the myosin is probably a true ATPase, splitting only the terminal phosphate from ATP, is indicated in the progress curves (Fig. 3). At the end of 60 minutes approximately 40% of the available "7 min." P had been hydrolyzed (Fig. 3A). If at the end of 40 minutes more substrate is added, additional inorganic phosphorus is released (Fig. 3B), indicating that the enzyme is still active. The enzyme, however, is rapidly inactivated when it is incubated, without substrate, at 37° C. for as little as 10 minutes: 97% of its ATPase activity is lost. It was also noted that much of the activity is lost if the squid are not freshly killed prior to extraction.

Inosinetriphosphate was actively hydrolyzed by squid myosin; Q_P of 1005. This value is not a completely accurate estimate of the ITP hydrolysis since the few experiments performed may reflect a lack of substrate saturation of the enzyme possibly due to high inosinediphosphate content: *i.e.*, at the end of 30 minutes the enzyme preparation had hydrolyzed only 14–16% of the total "7 min." P available (Fig. 3C).

On the assumption that the enzyme hydrolyzed all of the terminal phosphate present in the ATP preparations, the results (in Fig. 3A and of other experiments) indicate that approximately 40% of the "7 min." P is due to the terminal labile group of ATP, or that the substrate preparation contains about 80% ATP. The calculations of the Michaelis-Menten constant were, therefore, based on ATP concentrations calculated as 80% of the total "7 min." P. By this method K_m values, ranging from 2.58 to 3.63×10^{-4} with an average for 5 separate determinations of 3.05×10^{-4}, were obtained.

Discussion

Apparently squid mantle muscle lacks a magnesium-activated, water-soluble or particle-bound, ATPase comparable to that found in vertebrate striated muscle (rat, Kielley and Meyerhof, 1948; de Villafranca, 1954: rabbit, Perry, 1952: or pigeon, Kitiyakara and Harman, 1953) and in the muscle of many invertebrates such as the locust (Gilmour and Calaby, 1952), the oyster (Humphrey, 1949), and, very probably, the cockroach and housefly (Sacktor *et al.*, 1953; Sacktor, 1953). A small amount of this enzyme, too small an amount to be detected under the present conditions, might be present in squid mantle muscle, but its contribution to the total ATPase activity, as compared with the contribution made by myosin-ATPase activity, is negligible. This contrasts strongly with the situation Gilmour and Calaby report for locust muscle where the Mg-activated apyrase dominates the ATPase activity of the whole muscle, particularly in the highly active flight muscles.

The major portion of squid mantle ATPase activity exhibits much the same response to ions as vertebrate (Mommaerts and Seraidarian, 1947; de Villafranca, 1954) and other invertebrate myosin-ATPase; strong activation with calcium (approximately 10^{-2} *M*) and no activation to slight activation with magnesium. In contrast, myosins from the molluscs, *Mya arenaria* (Humphrey, 1948) and *Sepia officinales* (Nguyen-van-Thoai and Pin, 1950), both exhibit greater activity with magnesium than with calcium. It should be remembered, however, that mag-

nesium activation is also a characteristic of undissociated rabbit actomyosin (Szent-Gyorgyi, 1947).

Although the pH optimum for *Loligo* myosin-ATPase is considerably more acid than the optima for mammalian myosin (pH 6.2–6.5 and pH 9.2, Mommaerts and Seraidarian, 1947) it is comparable to the optima reported by Nguyen-van-Thoai and Pin (1950) for other molluscs: pH 5.8 for *Aplysia depilans, Pecten maximus,* and *Sepia officinales.* More alkaline optima were reported for *Mya arenaria* (pH 8.5, Humphrey, 1948) and for *Saxostrea commercialis* (just below pH 8, Humphrey, 1949). Since the unique effect of different buffers on myosin-ATPase activity is well known (Mommaerts and Seraidarian, 1947; Bailey, 1942), it is possible that the acid optimum (pH 5.9) reported here might be a function of the buffer used. Veronal buffer, however, showed no alkaline optimum. Experiments comparing the activity in histidine buffer, glycine buffer and "Tris" buffer at pH 8.8 to 9.0, revealed activity to decrease in that order. The apparent activating effect of histidine is being studied at this time.

It is interesting to note that the specific activity of squid myosin-ATPase is of the same order of magnitude as reported for mammalian myosin; Q_P-s ranging from 1000–6000 (Bailey, 1942). The values here (1000–2000) may be contrasted with the Q_P values reported by Humphrey (1948; 1949) which ranged from 9 to 60 for other invertebrates. The low values reported by Humphrey might be due to inactivation of the enzyme by the high temperatures, 35–38° C., at which it was tested. The squid enzyme, as stated previously, was quickly inactivated at temperatures of that order and the high Q_P values were obtained at 25° C.

Evidently the metabolism of squid muscle deviates somewhat from that of other animals. It would be of more than passing interest to ascertain whether the oxidative enzymes of the tricarboxylic acid cycle, found bound to the particles of mammalian muscle cells, were similarly bound in squid muscle. It would also be of interest to compare the function and chemistry of this muscle, which is composed of smooth muscle fibers (Dahlgren and Kapner, 1908; and the author's own unpublished observations), with striated muscle in an endeavor to clarify the real significance of striations.

Summary

1. It is highly probable that the mantle muscle of the squid, *Loligo pealii,* has only one ATPase, associated with the myosin-like proteins extracted with 0.5 *M* KCl. It lacks a water-soluble, or particulate-bound, ATPase comparable to that found in vertebrate and certain insect muscle.

2. The extremely labile myosin-ATPase is activated almost 10-fold by 0.01 *M* $CaCl_2$ and almost 4-fold by 0.001 *M* $MnCl_2$. Magnesium is without effect. The pH optimum for this enzyme is approximately pH 5.9. A true ATPase, being specific for the terminal phosphate of ATP, it also hydrolyzes ITP at a somewhat reduced rate. An average Michaelis-Menten constant of 3.05×10^{-4} and a Q_P as high as 2000, comparable to mammalian myosin-ATPase and much higher than other marine invertebrates, have been obtained.

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